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Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors

[steroid receptor/poly(amino acid) motif/homeotic gene/prostate]

CHAWNSHANG CHANG, JOHN KOKONTIS, AND SHUTSUNG LIAO

Ben May Institute, and Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637

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ABSTRACT **Structural analysis of cDNAs for human and rat androgen receptors (ARs) indicates that the amino-terminal regions of ARs are rich in oligo- and poly(amino acid) motifs as in some homeotic genes. The human AR has a long stretch of repeated glycines, whereas rat AR has a long stretch of glutamines. There is a considerable sequence similarity among ARs and the receptors for glucocorticoids, progestins, and mineralocorticoids within the steroid-binding domains. The cysteine-rich DNA-binding domains are well conserved. Translati n of mRNA transcribed from AR cDNAs yielded 94- and 76-kDa proteins and smaller forms that bind to DNA and have high affinity toward androgens. These rat or human ARs were recognized by human autoantibodies to natural ARs. Molecular hybridization studies, using AR cDNAs as probes, indicated that the ventral prostate and other male accessory organs are rich in AR mRNA and that the production of AR mRNA in the target organs may be autoregulated by androgens.**

Steroid hormones regulate differentiation and induce various physiological responses in eukaryotic organisms. These hormonal actions appear to require binding of steroid hormones to specific high-affinity receptor proteins in target cells (1–3) and interaction of the steroid hormone–receptor complexes with regulatory elements of specific genes (4). Molecular cloning and structural analysis of cDNAs for various steroid receptors in recent years have made it possible to analyze the importance of different receptor domains in binding DNA and steroid hormones.

Androgen receptors (ARs) are found in many androgen-sensitive organs such as the prostate, seminal vesicles, hair follicles, sebaceous and preputial glands, levator ani muscle, and various androgen-sensitive tumors (3). The rat ventral prostate has been used often to study various biochemical properties and intracellular dynamics of ARs (5, 6). Some abnormalities of the androgenic response may be due to mutations in AR genes (7).

We reported recently the characterization of cDNAs for human AR (hAR) and described androgen-binding properties of a 76-kDa protein encoded by a composite hAR cDNA (8). Other investigators have also succeeded in isolating a hAR cDNA that encodes a 41-kDa protein with specific androgen-binding properties (9). We report here the strategy we used to construct full-length hAR and rat AR (rAR) cDNAs, and we compare the nucleotide and the deduced amino acid sequences of hAR and rAR with the primary structures of other steroid receptors.

MATERIALS AND METHODS

cDNAs for rAR were obtained from λgt11 cDNA libraries constructed with poly(A)$^+$ RNA from rat ventral prostate (10) by methods described elsewhere for the construction of hAR cDNA (8). The rat cDNA library was screened with a 5′ ^{32}P end-labeled 41-base-pair oligonucleotide probe highly homologous to the DNA-binding domain of glucocorticoid receptor (GR). After eliminating clones for other steroid

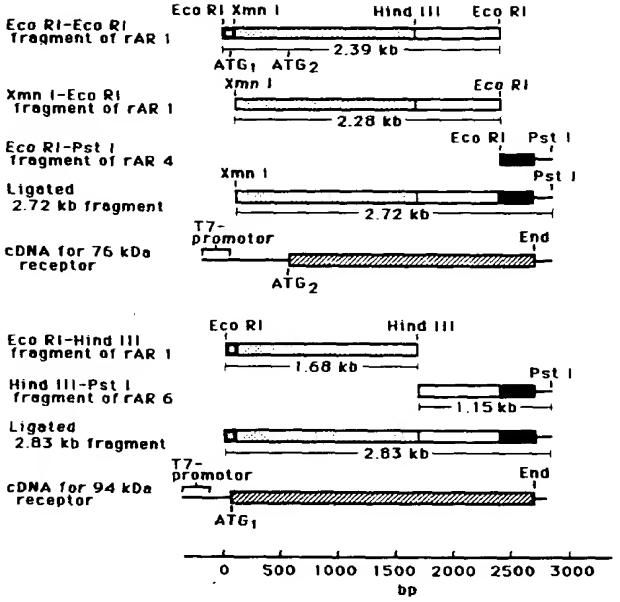

FIG. 1. Strategy used in the construction of cDNA for rAR. Three overlapped clones (rAR 1, rAR 4, and rAR 6) were used. To obtain the 2.72-kb cDNA that encoded a 76-kDa receptor protein, the 2.39-kb *Eco*RI/*Eco*RI cDNA insert of rAR 1 was digested with *Xmn* I to obtain a 2.28-kb fragment. This 2.28-kb *Xmn* I/*Eco*RI fragment was ligated to a 0.44-kb fragment obtained by digestion of the *Eco*RI/*Eco*RI cDNA insert of rAR 4 clone with *Pst* I. To obtain the 2.83-kb cDNA that could encode a 94-kDa receptor protein, the 2.39-kb *Eco*RI/*Eco*RI cDNA insert of rAR 1 was digested with *Hin*dIII to obtain a 1.68-kb fragment. This 1.68-kb *Eco*RI/*Hin*dIII fragment was ligated to a 1.15-kb DNA fragment that was obtained by digestion of the cDNA insert of rAR 6 clone with *Hin*dIII and *Pst* I. The ligated 2.72-kb construct was inserted into a *Sma* I and *Pst* I-digested pGEM-3Z vector, whereas the 2.83-kb construct was inserted into *Eco*RI and *Pst* I-digested pGEM-3Z vector. These vectors were used to transform *Escherichia coli* (DH5α) and transformed clones were selected by ampicillin resistance. The two 5′ end initiator (ATG$_1$ and ATG$_2$) and restriction enzyme sites are indicated. The open reading frame from the 5′ end ATG to the 3′ end is in hatched boxes. bp, Base pairs.

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Abbreviations: AR, androgen receptor; ER, estrogen receptor; GR, glucocorticoid receptor; PR, progestin receptor; MR, mineralocorticoid receptor; RAR, retinoic acid receptor; TR, thyroid receptor; h, human; r, rat; DHT, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); [^{3}H]R 1881, 17β-hydroxy-17-[^{3}H]methylestra-4,9,11-trien-3-one.

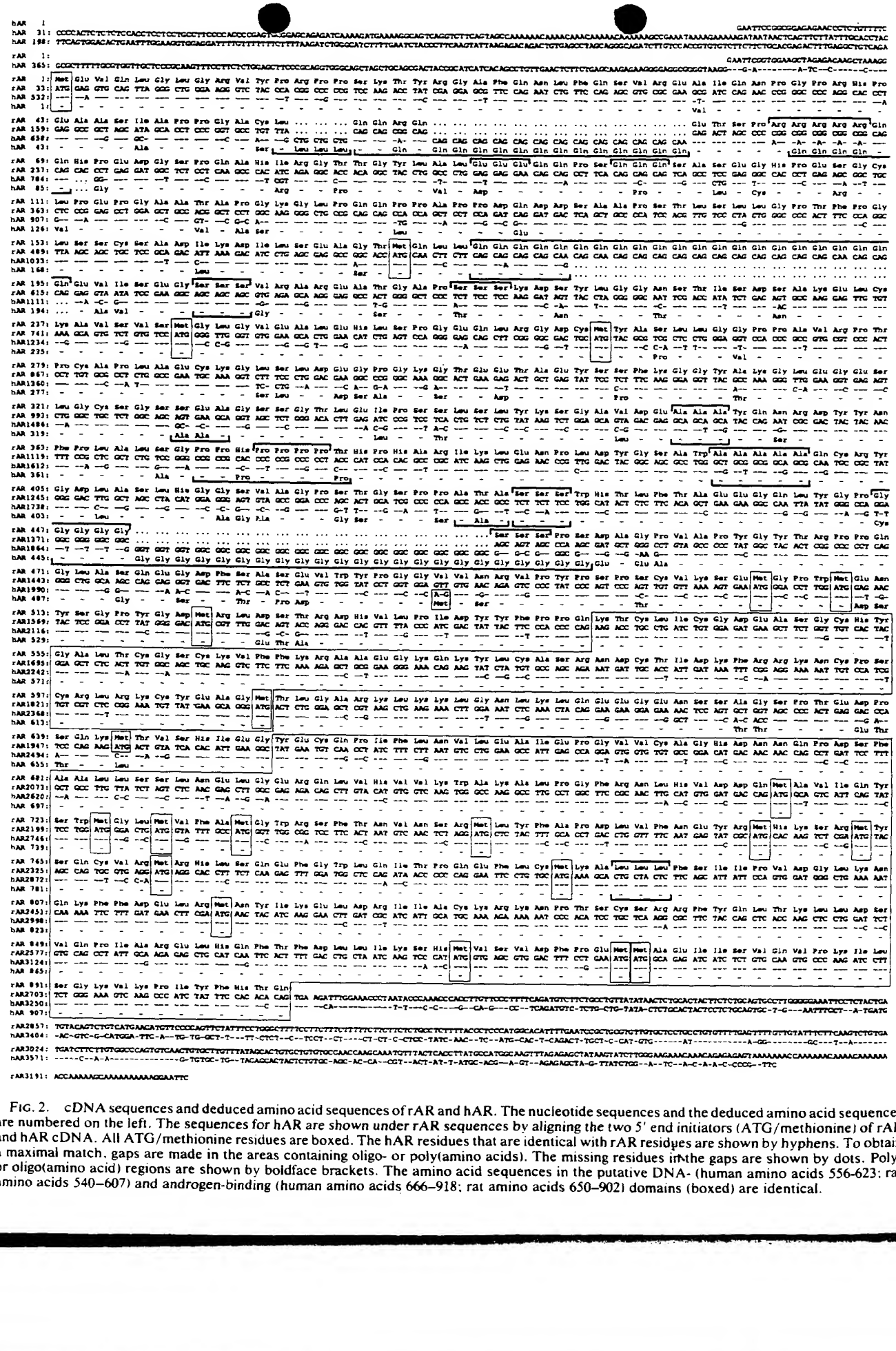

FIG. 2. cDNA sequences and deduced amino acid sequences of rAR and hAR. The nucleotide sequences and the deduced amino acid sequences are numbered on the left. The sequences for hAR are shown under rAR sequences by aligning the two 5′ end initiators (ATG/methionine) of rAR and hAR cDNA. All ATG/methionine residues are boxed. The hAR residues that are identical with rAR residues are shown by hyphens. To obtain a maximal match, gaps are made in the areas containing oligo- or poly(amino acids). The missing residues in the gaps are shown by dots. Poly- or oligo(amino acid) regions are shown by boldface brackets. The amino acid sequences in the putative DNA- (human amino acids 556–623; rat amino acids 540–607) and androgen-binding (human amino acids 666–918; rat amino acids 650–902) domains (boxed) are identical.

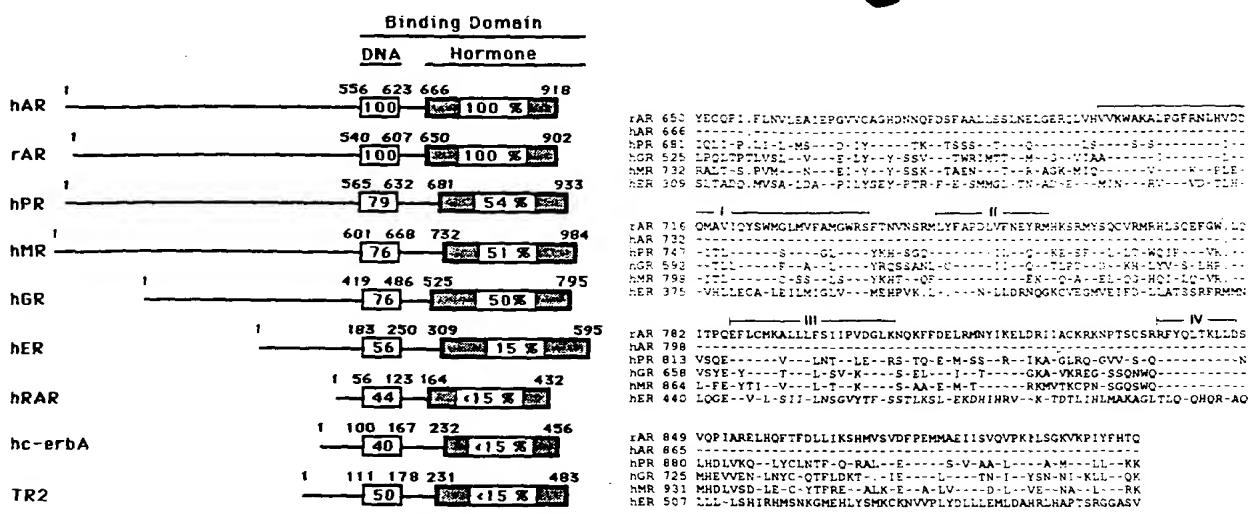

FIG. 3. Amino acid sequence homology among various steroid receptors. (*Left*) Homology at the DNA- and hormone-binding domains. The percentage similarity between AR and other receptors at each domain is shown inside boxes. The numbers outside the boxes represent the amino acid positions in the receptor sequences, starting from the N-terminal methionine of each receptor. TR2 is encoded by a cloned cDNA isolated from a human testis cDNA library (8). The natural ligand for TR2 is not known. (*Right*) The amino acid sequences (single-letter code) of the putative steroid-binding domains of various steroid receptors. The amino acid residues that are identical with that of hAR (or rAR) are shown by hyphens. Gaps (shown by dots) were made to allow the best matches. The amino acid position from the N-terminal methionine is shown on the left. Four homologous regions (I–IV) are shown.

receptors, the DNA inserts in the remaining clones were analyzed by restriction enzyme mapping and sequencing. Overlapping clones were selected, ligated, and inserted into pGEM-3Z vectors (Fig. 1). For construction of a full-length hAR cDNA, a 1.7-kilobase (kb) 5′ end hAR cDNA fragment was obtained by *Eco*RI digestion of a hAR cDNA clone isolated from the human prostate λgt11 cDNA library. The 1.7-kb fragment was digested with *Nru* I to give a 1.6-kb *Eco*RI/*Nru* I fragment that was then ligated to a 2.0-kb hAR fragment obtained by *Bam*HI and *Nru* I digestion of the 2.6-kb hAR in pGEM-3Z (8). The resulting 3.6-kb fragment was ligated into *Eco*RI and *Bam*HI-digested pGEM-3Z in the same orientation as the 2.6-kb hAR segment. The dideoxy chain-termination method (11) was used for nucleotide sequencing.

RESULTS AND DISCUSSION

Overall Structures. The nucleotide sequences and the deduced amino acid sequences of hAR and rAR cDNAs are shown in Fig. 2. The open reading frames of the two AR cDNAs, from the first ATG (at human nucleotide 532 or rat nucleotide 33) to the terminator TGA, encode 918 and 902 amino acids, respectively, for hAR and rAR with a molecular mass of ≈94 kDa. The first ATG is preceded by a terminator (TAA or TAG), indicating that it may be a natural initiator. The open reading frames from the second ATG (at human nucleotide 1084 or rat nucleotide 500) encode 734 and 733 amino acids, respectively, for hAR and rAR with a mass of ≈76 kDa. Since the nucleotide sequences surrounding these two ATG positions in AR cDNAs match Kozak's consensus sequence for an active start codon (ANNATGN), both the 76- and the 94-kDa forms of AR may be produced from a single gene in target organs. The existence of multiple forms of AR in normal androgen-sensitive organs has been suggested (3, 14).

DNA- and Androgen-Binding Domains. The primary structures of ARs deduced from AR cDNAs show that there is a cysteine-rich domain in hAR and rAR. The two ARs have an identical amino acid sequence in this domain but there are 14 nucleotide changes. The 68-amino acid region may fold into two "zinc coordinated finger" structures and bind to DNA as in other steroid receptors (15). All 10 cysteines of rAR, hAR, human progestin receptor (hPR) (16), and hGR (17, 18) as well as 9 of 10 cysteines in human estrogen receptor (hER) (19, 20) are conserved. A high homology (76–79%) is found between AR and hPR (16), hGR (17, 18), or human mineralocorticoid receptor (hMR) (21) (Fig. 3 *Left*). The homology between AR and hER (19, 20), human retinoic acid receptor (hRAR) (22, 23), chicken vitamin D receptor (24), and human thyroid receptor (hTR) or the oncogene product of avian erythroblastosis v-*erbA* virus (25) at this domain was within 40–56%.

The sequences of 253 amino acids at the C-terminal ends of hAR and rAR are identical, although there are 54 nucleotide changes. This region corresponds to the putative hormone-binding domain in other steroid receptors. In this region, the percentage similarity between AR and hPR, hGR, or hMR is 50–54%, whereas the similarity between AR and hER, hTR, or hRAR is insignificant (Fig. 3 *Left*). A feature of all steroid receptors is the high methionine content in their steroid-binding domains. For ARs, there are 13 methionines in this domain.

The amino acid sequence within the steroid-binding domain of rAR or hAR contains four highly conserved regions (Fig. 3 *Right*). The percentage similarity in these regions among human AR, PR, GR, and MR is 65–100%. The similarity between AR and ER at these regions is only 20–33%. It has been suggested that region I of GR is a potential signal transducing site and is involved in the intracellular activation/repression of steroid receptors (26).

The least conserved region in the C-terminal half is located between DNA- and steroid-binding domains. Between hAR and rAR, 6 of the 41 amino acids in this region are different. Since this hinge region is hydrophilic and is rich in both basic and acidic residues, it may be suitable for an ionic interaction between a receptor and a modulator. It has been suggested that this domain is important for ER-mediated inhibition of prolactin gene expression (27).

Amino Termini and Poly(Amino Acid) Motifs. Nonhomologous structures in various receptors may be important for receptors to carry out species- or organ-specific functions and/or may be involved in differential modulation of receptor activity. In fact, the N-terminal regions in different steroid

receptors do not appear to have a uniform structure. The differences in molecular sizes among different steroid receptors are largely due to the differences in the peptide length at these regions (Fig. 3 *Left*).

In the N-terminal region, 116 of 555 amino acids of hAR are not identical with the corresponding sequences in rAR (Fig. 2). The most striking feature of AR cDNA, however, is the presence of different repetitive nucleotide sequences that encode oligo- or poly(amino acid) sequences in the N-terminal regions. hAR cDNA has consecutive CAG/CAA triplets encoding a stretch of 17 glutamines and consecutive GGC/GGT triplets encoding a stretch of 27 glycines, whereas rAR cDNA has consecutive CAG/CAA triplets encoding a stretch of 22 glutamines. In addition, hAR also has a tetramer of leucine, an octamer of proline, and a hexamer and a pentamer of glutamine, whereas rAR has an pentamer of arginine, a pentamer of glycine, a trimer of glutamic acid, a trimer of glutamine, and a tetramer of proline. Both ARs also have trimers or pentamers of alanine and serine. A trimer of leucine was present in the androgen-binding domain of hAR and rAR; otherwise, no other oligomeric amino acid is present in the DNA- or hormone-binding domains of ARs.

Many regulatory gene products are known to contain poly(amino acid) sequences. Among the most interesting examples are the "pen" or GGN repeat (28), which encodes polyglycine, and the "opa" (or "M") or CAG repeat (29), which encodes polyglutamine in several homeotic genes (30). A glutamine stretch was also found in rGR (18), but, to our knowledge, no other steroid receptor cDNA that has been analyzed so far has a nucleotide sequence that encodes a long stretch of a poly(amino acid). The role of poly(amino acid) sequences in homeotic genes and steroid receptors is not clear. It is conceivable that they may participate in the interaction of receptor proteins with components of transcription machinery or with modulatory factors. The presence of these protein motifs in some steroid receptors supports the suggestion that many genes with different roles in the control of development may have evolved from the same ancestral gene (30).

Transcription and Translation. By $NaDodSO_4$/PAGE analysis, we found that the 2.6-kb hAR cDNA yielded a major translation product that migrated as a 76-kDa protein (8), whereas the 2.8-kb rAR cDNA and a 3.6-kb hAR cDNA produced not only a 94-kDa protein but also 76-, 70-, 55-, 46-, 32- and 30-kDa proteins (Fig. 4). Similar protein products were obtained from the 2.7-kb rAR cDNA, although a 93-kDa protein (instead of a 94-kDa protein) was obtained reproducibly. All of these products were immunoprecipitable by a human serum containing autoimmune antibodies to AR (31) and were able to bind DNA, indicating that each of them had a DNA-binding domain.

The production of the 94-, 76-, 70-, and 46-kDa proteins can be explained as products of translation initiation at the first and other internal ATG codons at the amino acid positions (based on hAR sequence) 1, 185, 241, and 522 (also 526 or 536). The 55-, 32, and 30-kDa peptides may be proteolytic products of large forms. Since the 2.7-kb rAR cDNA should not encode a protein larger than 76 kDa, the identity of the 93-kDa protein produced from this cDNA is not clear.

Androgen Binding and Receptor Characterization. AR made in a reticulocyte lysate bound 17β-hydroxy-17-[^{3}H]-methyl-estra-4,9,11-trien-3-one ([^{3}H]R 1881) specifically. The apparent dissociation constants based on Scatchard plot analysis (13) for hAR and rAR were 0.3 ± 0.1 nM (SEM) and 0.5 ± 0.3 nM (SEM), respectively. 5α-Dihydrotestosterone (DHT), a potent natural androgen, as well as another potent synthetic androgen, 7α,17α-dimethyl-19-nortestosterone (12), competed very well, whereas 17β-estradiol, progesterone, and dexamethasone were not effective competitors (Table 1). Testosterone was also much less effective than

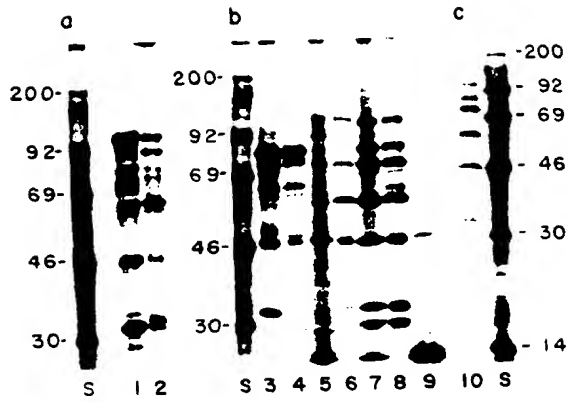

FIG. 4. PAGE analysis of ARs made from cDNAs. Linearized pGEM-3Z vectors containing AR cDNA segments were transcribed by T7 RNA polymerase and RNA made was translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The lysate mixture containing proteins synthesized from the 3.6-kb hAR cDNA (lanes 1 and 2), 2.6-kb hAR cDNA (lanes 3 and 4), 2.83-kb rAR cDNA (lanes 5 and 6), 2.72-kb rAR cDNA (lanes 7, 8, and 10), and control (without cDNA; lane 9) were analyzed by $NaDodSO_4$/PAGE. Lanes 2, 4, 6, and 8, proteins were immunoprecipitated with a human serum containing autoantibodies to rAR or hAR (31) before the analysis. Lane 10, the lysate was passed through a DNA-cellulose column to absorb DNA-binding proteins. DNA-bound proteins were eluted from the column by a 0.4 M KCl solution and analyzed (12, 31). Lane S, size standards including lysozyme (14 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (69 kDa), and phosphorylase b (92 kDa). The concentration of acrylamide gel used for PAGE was 8% in *a* and *b* and 10% in *c*.

DHT or R 1881. This is in line with the suggestion (32, 33) that DHT, rather than testosterone, is the active form of androgens in prostate and in some other target organs. By estimating the amounts of AR made from rAR cDNA, the size distribution (Fig. 4), the number of methionine residues in each form (as predicted from sequence structure), and binding of [^{3}H]R 1881 at a saturating ligand concentration, each of the AR molecules that was larger than 32 kDa appeared to bind 1.0 ± 0.2 molecule of androgen, indicating that most of the newly synthesized AR peptides have the androgen-binding domain.

In a low salt medium, various steroid-receptor complexes sediment as 3–5S, 7–10S, or even heavier forms (1–3). The 7–

Table 1. Androgen-specific binding of rAR encoded by cloned cDNA

Unlabeled steroid added	[^{3}H]R 1881-bound, % of control	
	50 nM	500 nM
R 1881	19	3
DMNT	21	3
Testosterone	66	16
5α-Dihydrotestosterone	30	10
17β-Estradiol	100	90
Progesterone	85	82
Dexamethasone	99	95

RNA transcribed from the cloned 2.72-kb cDNA (see Fig. 1) was translated in a rabbit reticulocyte lysate system. Aliquots of the lysate were then incubated with 5 nM [^{3}H]R 1881 (87 Ci/mmol) in the absence (control) or presence of unlabeled steroids (50 or 500 nM). The final incubation vol was 100 μl. [^{3}H]Androgen binding was measured by the hydroxylapatite filter method (12). The result was expressed as a percentage of the label bound in the control tube (2500 dpm) without additional unlabeled steroid. The experiment was repeated twice to ensure reproducibility. The values from duplicate assays were within 10% of the average shown in the table. DMNT, 7α,17α-dimethyl-19-nortestosterone.

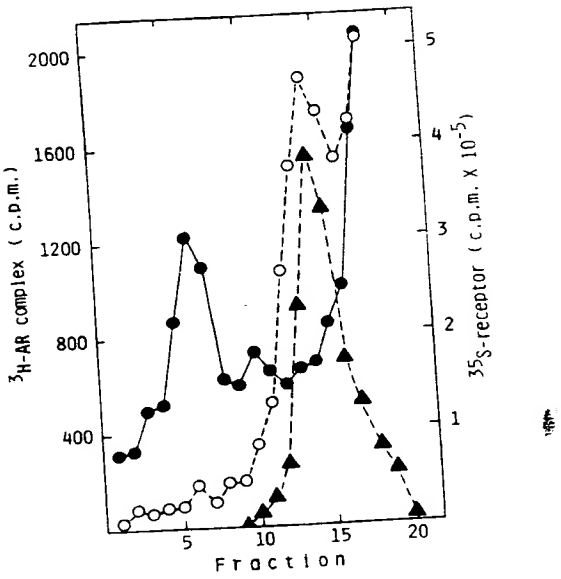

FIG. 5. Gradient profiles of hAR synthesized in a cell-free system. hAR made in a cell-free system using the 2.6-kb hAR cDNA in the presence of [^{35}S]methionine (▲) (see Fig. 4). hAR made in the same way in the presence of unlabeled methionine and allowed to form a complex with [^{3}H]R 1881 (○), and the [^{3}H]R 1881–receptor complex incubated with a human serum containing autoantibodies to hAR (●) were analyzed by gradient centrifugation (31). Centrifugation was performed at 34,000 rpm for 16 hr at 2°C in a Beckman SW 60 rotor. Fractions (200 μl each) were collected and numbered from the bottom of the tube. Bovine serum albumin (4.6S) sedimented in the vicinity of fraction 13.

10S or larger forms can dissociate into the 3–5S form in a medium containing 0.4 M KCl. In a medium containing 0.4 M KCl, ^{35}S-labeled AR made from hAR cDNA sediments as a 4S form (Fig. 5). Omission of KCl from the medium or addition of a cytosolic fraction of rat ventral prostate to the ^{35}S-labeled hAR preparation did not alter the sedimentation pattern of the radioactive AR, suggesting that the 7–10S or larger forms of AR may be formed only after a modification of the newly made hAR and/or an association with a specific macromolecule. [^{3}H]R 1881-bound hAR also sediments at 4S in a high salt medium (Fig. 5). As expected, the radioactive androgen-receptor complex interacted with autoantibodies to AR and sedimented as 8–10S.

AR mRNA Levels and Androgen Regulation. To analyze the level of AR mRNA in various organs, poly(A)$^+$ RNA was isolated and analyzed by dot hybridization. Hybridization was carried out with a nick-translated 2.4-kb *Eco*RI/*Eco*RI fragment of plasmid rAR 1 (Fig. 1) under conditions of high stringency, and hybridized radioactivity was determined (10). The results indicated that, among androgen-sensitive rat organs tested, the level of AR mRNA-like sequences per unit amount of poly(A)$^+$ RNA was highest in seminal vesicle, ventral prostate, and coagulating gland. Kidney and levator ani muscle were ≈20%, whereas thigh muscle was <10% of the level found in seminal vesicle (data not shown).

We have used adult Sprague–Dawley male rats to analyze the effect of androgens on AR mRNA levels in rat ventral prostate. Using the same hybridization method, we found that AR mRNA levels per unit of poly(A$^+$) RNA increased to 140% of normal 2 days after castration. This effect was reversed completely by the daily injection of castrated rats with DHT (5 mg per rat per day). This experiment suggested that androgens may be able to autoregulate the level of AR mRNA in their target organs.

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